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Responses of succinate dehydrogenase and non-specific alkaline phosphatases and mortality of tilapia to ambient *p*H stress in a sewage-fed aquaculture pond

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The fish, tilapia (*Oreochromis mossambicus*) of 50-60 g body weight was experimentally exposed to effluent gradients of highly alkaline *p*H in a sewage-fed aquaculture farm for examining the *p*H stress-induced responses of mortality and the stress marker enzyme succinate dehydrogenase and the non-specific alkaline phosphatases of fish prior to death at different hours of intoxication. A second trial was performed after two months when water quality changed along the sewage effluent gradient. An *in situ* experiment was also performed for better understanding of the responses of enzymatic activities attributable to different levels of *p*H conditions. Time required for 100% mortality of fish tended to increase from 30 min in *p*H 11.6 to 22 hr in *p*H 10.2. There was no mortality of fish when water quality improved significantly (with *p*H ranging between 9.6 to 8.0) after two months. The activities of succinate dehydrogenase and intracellular alkaline phosphatases assayed in gills and liver prior to death of fish tended to reduce with increase in survival hour, following a pattern of decay curve. On the other hand, percent of enzymatic inhibition of the exposed fish over the control increased as the survival hour increased following a pattern of exponential curve. It appears that the highest water *p*H of 11.6, maximum ratio for ammonium to ammonium hydroxide (1: 21) and reduced level of dissolved oxygen (2.62 mg/l) were perhaps responsible for the 100% mortality of fish within 30 min of their exposure and the enzymatic activities in the gills and liver assayed prior to death of fish tended to reduce as the acclimatization period of fish increased and *vice-versa.*

Keywords: Alkaline phosphatase, Ammonia toxicity, Fish mortality, *p*H, Succinate dehydrogenase, Stress

Fishes are primary aquatic vertebrates using gills for respiration. Changes in water quality such as *p*H, decrease in dissolved O_2, presence of toxic substances, cumulative effects of pesticides and various other compounds are some of the major causes of respiratory distress to fish especially those living in the wastewater-fed aquaculture system with relatively high level of environmental stressors. The stressors in the environment exert their adverse effects at the organismal level leading to impaired physiological functions and eventually death of fish.

Krebs cycle functioning in the inner membrane of mitochondria is the major degradative pathway for the generation of ATP[1]. The mitochondrial respiratory enzyme succinate dehydrogenae (SDH) is a primary enzyme in the oxidative catabolism of sugars[2] and as such is used effectively as a marker of mitochondrial abundence and activity. This enzyme is concentrated in the chloride cells within fish gills and has been used as an indicator of the osmoregulatory activity[3,4]. Exposure of fish tilapia, *Oreochromis mossambicus* to sublethal acid water resulted in structural damages and death of chloride cells in the gills by necrosis, but the ion transport capacity of gills was increased slightly[5]. Artificial exposure of adult or fingerling of *Labeo rohita* at lethal or sublethal concentrations of lead and copper or organochlorine (DDT and BHC) and organophosphorous (Dichlorvos and Monocrotophos) compounds resulted in gradual decrease in SDH activity in different tissues (gill, liver, muscle and brain) of fish over time[6-8].

Alkaline phosphatase is a P-stress marker enzyme that catalyses the hydrolysis of phosphorous compounds and the transfer of phosphoryl groups to an acceptor molecule. The rate of catalytic activity of the enzyme is inversely proportional to the concentration of inorganic phosphate in the ambient environments[9]. This enzyme could serve as a good indicator of intoxication because of its sensitivity to metallic salts[10].

Fishes living in the wastewater-fed ponds are constantly being exposed to varying levels of dissolved oxygen and *p*H of water caused by the presence of wide array of chemical pesticides with often enriched level of phosphorous in the waters. Hydro mineral disturbance has been stated to disturb

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the normal physiological functions of fishes. The external factors such as water *p*H, mineral composition and ionic calcium levels have significant impact on environmental quality of water. Hardness of water, however, may play an important role in reducing toxic effects of a metal through competitive inhibition at the gill surface[11].

In August 2005, an influx of highly alkaline effluents from some industries resulted in mass mortality of fish along the sewage effluent gradient of a sewage-fed fish farm in Kalyani (22.98°N; 88.48°E), West Bengal, India. This signaled alarm not only to government agencies but also to fisheries scientists who got an opportunity to investigate the water quality parameters of the pond that causes the fish death as well as the responses of some important stress marker enzyme system prior to death of fish. The purpose of the present study is to examine the causes of fish death in terms of water quality and the responses of some important stress marker enzyme (SDH and non-specific alkaline phosphatase) prior to fish death in response to different levels of highly alkaline *p*H along the sewage effluent gradient in a sewage-fed fish farm.

Materials and Methods

The study was conducted in a sewage-fed aquaculture farm at Kalyani, West Bengal. It consisted mainly of eight ponds: two anaerobic (26 m×52 m×2.5 m), two facultative (64 m×150 m×1.5 m) and four (1-4) stocking (52 m×156 m×1.0 m) placed serially. The two anaerobic ponds are connected with the two facultative ponds via inlets. Both the facultative ponds are connected with the first stocking pond through a common channel which is again connected with the remaining three stocking ponds via their outlets placed diagonally to each other. Thus the raw sewage water influxes into the anaerobic pond through an inlet and finally is disposed out to a canal outside the farm via an outlet of the last stocking pond[12,13].

The sewage system plant is connected by the underground sewer system fed mainly by domestic sewage of the town and is mixed occasionally by the effluents from some industries manufacturing battery, dye, pharmaceutical drugs, detergents, etc. Heavy metals from some industrial wastes and some chemicals from domestic sewage constitute the main source of pollution in this fish culture system. These pollutants are, however, diluted by rain water especially during the monsoon.

Three experiments were performed to examine the *p*H stress induced changes in SDH and alkaline phosphatases activity of tilapia prior to death *in situ*. Four sites (site-A to site- D) along the effluent gradient of facultative pond of a sewage fed fish farm were selected in first and second experiments, whereas the third experiment was designed to confirm the effects of *p*H induced fish mortality under simulated condition.

The design for first and second experiments were basically similar using four sites (site- A, site-B, site-C and site-D) in each. However, the environmental condition was more stressful during the first experiment (August, 2005) due to discharge of highly alkaline sewage effluents (*p*H ranged from 11.6-site-A to 10.2-site-D). Physicochemical examination of domestic raw sewage[12] of Kalyani township showed the presence of following characteristics: *p*H, 6.9-7.2; BOD, 120-360 (mg/l); COD, 390-720 (mg/l); dissolved oxygen, Nil; free carbon dioxide, 60-130 (mg/l); total alkalinity, 490-650 (mg/l); orthophosphate, 6-30 (mg/l); ammonia, 65 (mg/l); suspended solids, 160-520 (mg/l); lead, 0.149-3.870 (mg/l); chromium, 0.025-0.137 (mg/l) and cadmium, 0.004-0.018 (mg/l).

Adult and healthy tilapia (*Oreochromis mossambicus*) (50-60 g) collected from a local fish culture pond were acclimatized for 3-4 days in the outdoor tanks prior to experimentation. Five acclimatized fish were introduced into closed cage in quadruplicate and each cage was suspended in four different sites of the facultative pond. The second experiment was performed in the field after two months when there was significant improvement in water quality of the sewage effluent (*p*H values ranged from 9.6 at site-A to 8.0 at site-D). Other conditions remained same as that of first experiment.

The third experiment was performed in nine 50 l glass containers placed in the outdoor condition using well aerated ground water (*p*H 7.2). The *p*H of water was adjusted to three different levels: 8, 10, and 12. Each treatment had four replicates using $Ca(OH)_2$ as adjusting chemical to bring about the desired *p*H. The simulated water *p*H was confirmed by repeated *p*H measurement. Three healthy and acclimated tilapia (50-60 g) were introduced in each glass container at a specific time and were under constant observation till they died.

Surface water was collected from each site of the sewage effluent and were analysed for water quality e.g. temperature, *p*H, dissolved oxygen (DO),

ammonium-N (NH_4-N), nitrite-N (NO_2-N), nitrate-N (NO_3-N) and phosphate (PO_4-P) following the methods described in APHA[14].

The exposed fishes prior to their death (30 min-24 hr) were collected from all the experimental set up and were sacrificed for determination of the activities of succinate dehydrogenase and alkaline phosphatases from gills and liver. Groups of fish of identical weight collected from a local fish culture pond that served as control and were also sacrificed for enzymatic assay. The collected tissue samples were subjected to assay of the stress marker enzymes, succinate dehydrogenase and alkaline phosphatases following the standard protocols[15,16].

Arithmetic mean (±SE) of succinate dehydrogenase and alkaline phosphatases obtained from 3-5 samples was used in the present study. Exponential decay curve of succinate dehydrogenase activity against survival period was derived from the regression analysis package. The significance level was accepted at $P < 0.05$.

Results

Experiment-1

Mortality — Highly alkaline condition with *p*H 11.6 and sharp depletion in dissolved oxygen (2.62 mg/l) resulted in 100% mortality of the test fish within 30 min of their exposure at site-A of the sewage effluent gradient in facultative pond (Table 1). With gradual decline in the *p*H values and concurrent increase in dissolved oxygen concentration along the passage of the effluent gradient, the survival period of fish increased from 30 min (site-A) to 22 hr (site-D) of the facultative pond (Fig. 1).

Enzymatic assay — The SDH activity in both the tissues of fish was higher in the control group than those held in different sites of the facultative pond irrespective of the time of death. There was gradual decline in SDH activity in those fish held in different sites along the gradient of sewage effluent at different periods of survival (Fig. 2). The percent of reduction ranged between 26.92-89.51 and from 27.52-87.38 in case of gills and liver respectively.

The SDH activity observed at the time of death was much higher when the fish survived for a short duration than the one with longer duration. At site- A, the SDH in gills ranged between 0.00057-0.00034 mg/g/hr and 0.00029 to 0.00018 mg/g/hr in liver. The SDH activity assayed at the time prior to fish death tended to reduce gradually with increase in survival period of fish resulting in a pattern of decay curve (Fig. 2). The per cent inhibition of SDH with respect to control increased with survival periods following a of trend of an exponential curve.

The responses of alkaline phosphatases (Fig. 3) were identical to that of SDH. The reduction of activity of alkaline phosphatases in different tissues of exposed fish ranged between 59.15 to 93.63% in gill and 47.35 to 90.89% in liver in all the sites under investigation.

Water quality — The *p*H ranged between 11.6-10.2 at the time of death of fish in different locations of facultative pond. The concentrations of dissolved oxygen varied by a factor of 2.73 ranging between 2.62-7.16 mg/l. The ratio of NH_4 : NH_4OH was maximum (1:21) at site-A with *p*H 11.6 and minimum at site- D (1: 7) with *p*H 10.2 . The concentrations of nitrite (NO_2-N) and nitrate (NO_3-N) ranged between 0.003-0.0386 mg/l and between 0.0075-1.462 mg/l respectively. The amount of phosphate (PO_4-P) varied between 0.28 to 0.097 mg/l (Table 1).

Experiment-2

Mortality — With significant improvement in water quality after 2 months, there was no mortality of exposed fish in any of the four sites of the facultative pond with *p*H values ranging between 9.6 to 6.8 and dissolved oxygen between 6.18 to 10.2 mg/l (Table 1).

Enzymatic assay — There was gradual decline in the activities of both the enzymes SDH (Fig. 4) and alkaline phosphatases of fish held in different sites along the gradient of sewage effluent. The percent of reduction of both the enzymatic activities was higher in gills (78.98% and 89.41% for SDH and alkaline phosphatases respectively) than in liver (77.96% and 73.82% for SDH and alkaline phosphatases respectively) in all the four sites investigated. The reduction of SDH activity (gills–0.00064 mg/g/hr; liver-0.00031 mg/g/hr) was higher than the alkaline phosphatases (gills-15.42 nmole/min/g; liver-3.27 nmole/min/g) in both the tissues examined. The percent of reduction in enzyme activity with respect to control tended to increase as the survival period of fish increased following a trend of exponential curve for SDH (Fig. 5) and alkaline phosphatases.

Water quality — Water quality remained much better with variations of *p*H between 6.8 and 9.6 and dissolved oxygen between 6.18 and 10.2 mg/l in different locations during the period of study. The concentrations of nitrite (NO_2-N) and nitrate (NO_3-N) ranged between 0.122 mg/l to 0.183 mg/l and between

Table 1 — Mortality, SDH and alkaline phosphatases activity of tilapia and water quality in four sites of facultative pond examined in different experiments employed

Parameters	Expt.-1				Expt.-2				Expt.-3		
	Site-A	Site-B	Site-C	Site-D	Site-A	Site-B	Site-C	Site-D	Cont.-1	Cont.-2	Cont.-3
Mortality (%)	100	100	100	100	0	0	0	0	100	100	0
Survival period (hr)	0.5-3.0	3.0-5.0	7.0	21.0-22.0	>22.0	>22.0	>22.0	>22.0	0.12-0.16	19:45-20:15	>72.0
Enzymatic Assay											
SDH activity (mg/g/hr)											
Gill	0.00057-0.00034	0.00024-0.00019	0.00016	0.00009-0.00006	0.00045-0.0002	0.00026-0.00013	0.00011	0.00003-0.00002	0.00068-0.00056	0.00032-0.00017	0.00064-0.00047
Liver	0.00029-0.00018	0.00017-0.00013	0.00012	0.00010-0.00009	0.00021-0.00013	0.00011-0.0001	0.00008	0.00001-0.000009	0.00038-0.00022	0.00017-0.00009	0.00033-0.00024
Non-specific alkaline phosphatases (nmol/min/g)											
Gill	7.606-2.744	2.661-2.251	1.607	1.28-1.188	4.75-2.6	2.56-1.385	1.07	0.476-0.42	16.75-14.87	8.17-6.01	17.48-14.53
Liver	2.248-2.11	2.07-1.564	1.36	0.680-0.322	2.105-2.025	1.975-1.115	0.633	0.384-0.327	2.728-1.869	1.087-0.722	3.08-2.865
Water quality											
Temperature (°C)	36	35.2-35.6	34.8	33.5	28-33	27-33	28-31	29-33	30	30-30.2	29-30
*p*H	11.4-11.6	11.0-11.3	10.8	10.2	8.2-9.6	7.1-8.3	7.1-8.0	6.8-8.0	12	10	8
Dissolved oxygen (mg/l)	2.62-3.11	3.15-3.94	4.27	6.22-7.16	7.34-10.2	6.89-7.56	6.42-7.36	6.18-7.38	6.76-7.21	4.26-6.61	5.55-5.82
NH_4-N (mg/l)	0.0024-0.0195	0.0202-0.0255	0.0311	1.25-1.31	2.58-3.37	2.87-3.82	2.92-4.01	2.73-3.83	-	-	-
NH_4OH (mg/l)	0.05-0.37	0.37-0.39	0.49	8.74-9.16	7.52-13.35	6.64-11.50	6.44-11.05	5.65-10.27	-	-	-
NH_4: NH_4OH	1:19-1:21	1:15-1:18	1:13.1	1:7	1:4.9-1:2.9	1:3-1:2.2	1:2.7-1:2.07	1:2.75-1:2.07	-	-	-
NO_2-N (mg/l)	0.0064-0.003	0.0072-0.0145	0.0185	0.0335-0.0385	0.135-0.176	0.127-0.175	0.122-0.172	0.130-0.183	-	-	-
NO_3-N (mg/l)	0.0075-0.1102	0.1122-0.1694	0.368	1.314-1.462	0.116-0.183	0.116-0.183	0.139-0.187	0.116-0.170	-	-	-
PO_4-P (mg/l)	0.23-0.28	0.197-0.286	0.183	0.097-0.10	0.152-0.183	0.173-0.196	0.184-0.202	0.175-0.186	-	-	-

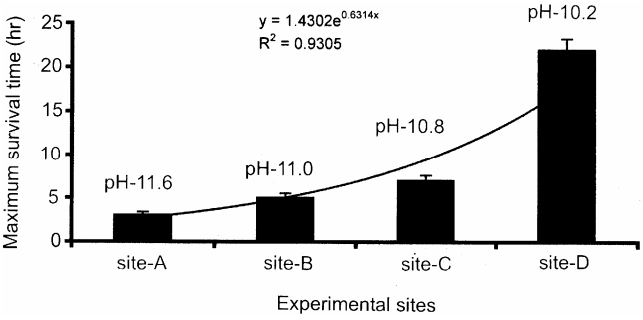

Fig. 1 — Time required for 100% mortality of tilapia in four sites along the sewage effluent gradient in facultative pond of Kalyani sewage fed fish farm (Experiment-1). Bar indicates the mean (± SE) of survival periods. Exponential equation was significant at $P < 0.05$.

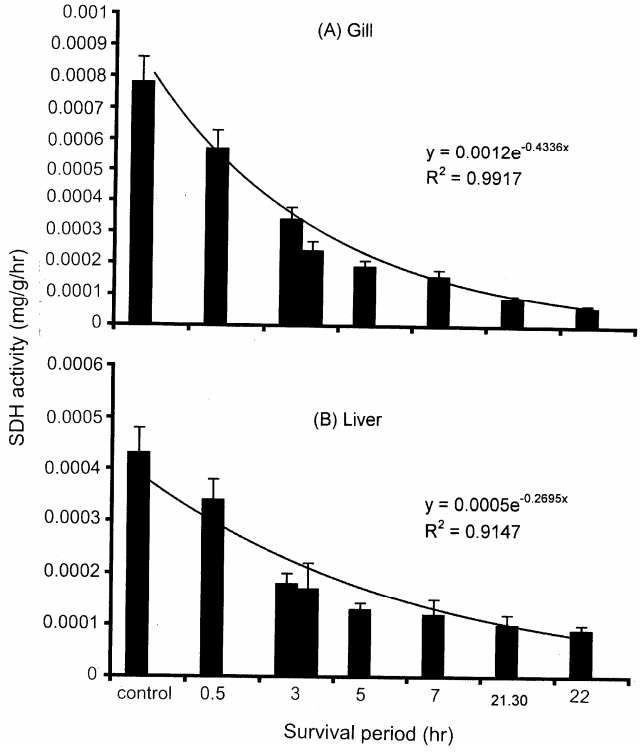

Fig. 2 — SDH activity in gill (A) and liver (B) in different survival periods of tilapia exposed at different sites of sewage effluent gradient (Experiment-1). The data of four different sites were used. Bar indicates the mean (± SE) of SDH activity of gill (A) and liver (B). Exponential equation was significant at $P < 0.05$.

0.116 mg/l to 0.187 mg/l, respectively. The amount of phosphate (PO_4-P) varied between 0.152 mg/l to 0.202 mg/l (Table 1).

Experiment-3

Mortality — Time required for 100% mortality of exposed tilapia varied markedly under different artificially created *p*H conditions between 8 and 12 of

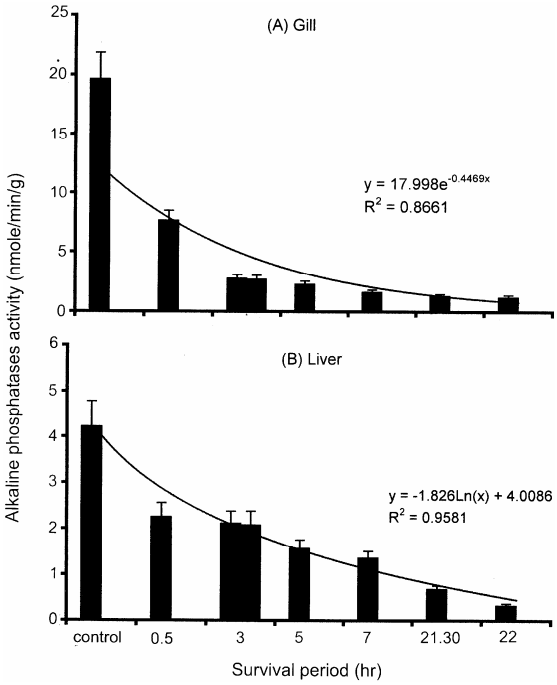

Fig. 3 — The alkaline phosphatases activity in gill (A) and liver (B) in different survival periods of tilapia exposed at different sites of the sewage effluent gradient (Experiment-1). The data of four different sites were used. Bar indicates the mean (± SE) of alkaline phosphatases activity of gill (A) and liver (B). Exponential equation was significant at $P < 0.05$.

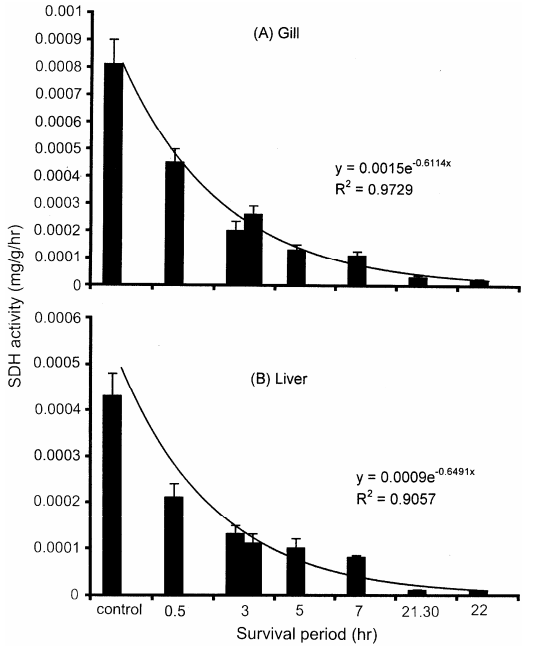

Fig. 4 — SDH activity in gill (A) and liver (B) in different survival periods of tilapia exposed at different sites of sewage effluent gradient (Experiment-2). The data of four different sites were used. Bar indicates the mean (± SE) of SDH activity of gill (A) and liver (B). Exponential equation was significant at $P < 0.05$.

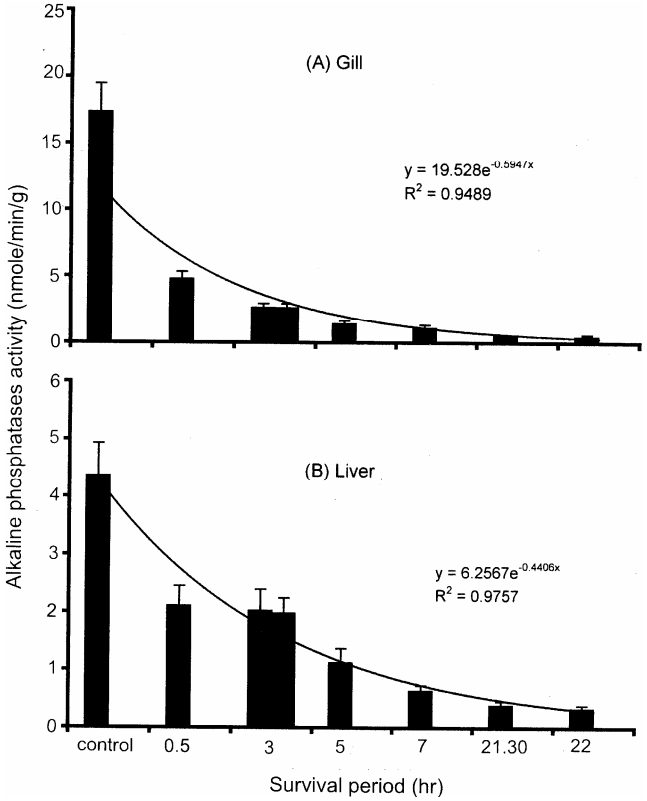

Fig. 5 — The alkaline phosphatases activity in gill (A) and liver (B) in different survival periods of tilapia exposed at different sites of the sewage effluent gradient (Experiment-2). The data of four different sites were used. Bar indicates the mean (± SE) of alkaline phosphatases activity of gill (A) and liver (B). Exponential equation was significant at $P < 0.05$.

water (Table 1). No mortality occurred at *p*H 8 whereas all the fish died between 19.45-20.15 hr and between 7-10 min when the fish faced *p*H of 10 and 12, respectively.

Enzymatic assay — Both, SDH (Fig. 6) and alkaline phosphatases (Fig. 7) activities in different tissues of tilapia held under experimental *p*H conditions were lower than the control group of fish held at water *p*H 7.2. The activities of SDH (0.0010 mg/g/hr) and alkaline phosphatases (12.23 nmole/min/g) were higher in the gills than those in the liver (SDH: 0.00023 mg/g/h and alkaline phosphatases: 2.03 nmole/min/g).

The SDH activity in gill tissue was reduced by 55.26% when death occurred within 7-10 min of exposure at simulated *p*H 12 and liver tissue showed 72% reduction during the same time period. With increase in survival time of fish at *p*H 10, there was further reduction in the activities for SDH and alkaline phosphatases in both gills and liver tissues.

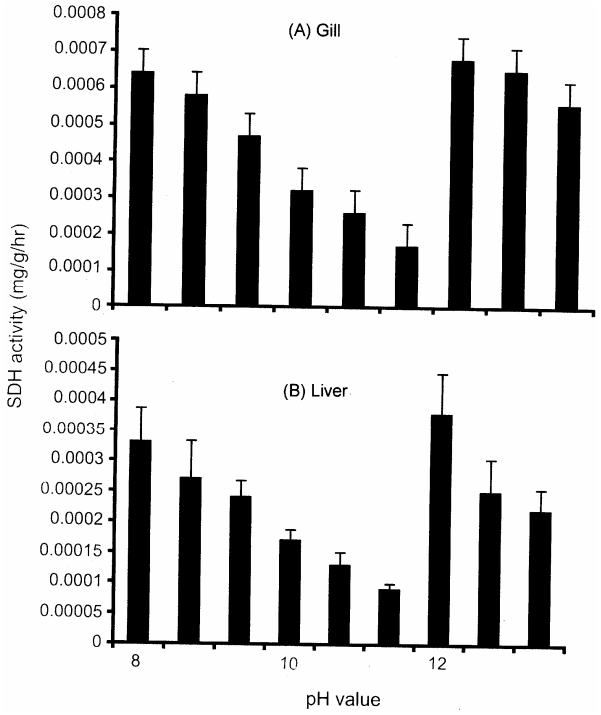

Fig. 6 — SDH activity in gill (A) and liver (B) of fish prior to death against different *p*H conditions (Experiment-3). Bar indicates the mean (± SE) of SDH activity of gill (A) and liver (B).

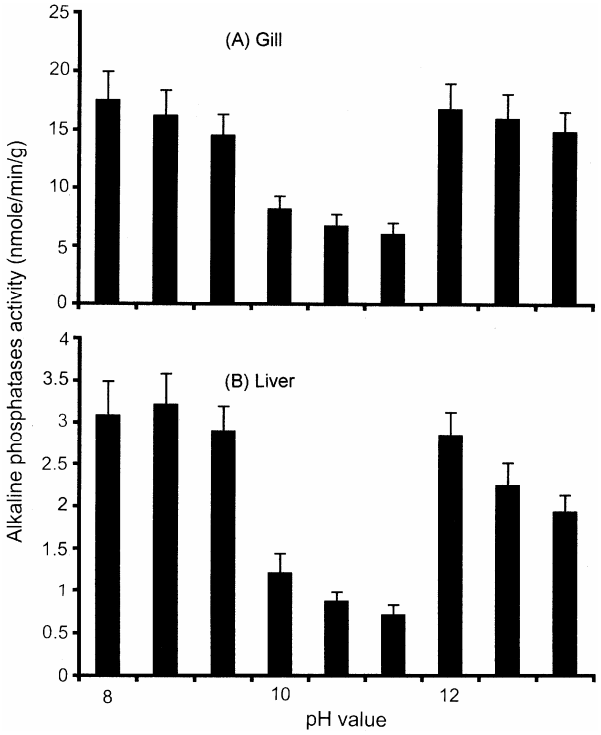

Fig. 7 — Alkaline phosphatases activity in gill (A) and liver (B) of fish prior to death against different pH conditions (Experiment-3). Bar indicates the mean (± SE) of alkaline phosphatases activity of gill (A) and liver (B).

Discussion

The present data reveal that the high alkaline *p*H of 10.2-11.5 of the sewage effluent was probably responsible for the death of fish by acting as a stressor, mediated through the chemical alterations that signal the physiological activities of fish. It is known that changes of *p*H, even by a small range, may cause a major stress in the form of chemical change; *p*H alternations of more than 0.3 units/day may act as *p*H shock[17]. As a result, maximum survival time of fish gradually increased from 30 min when exposed at *p*H 11.6 to 22 hr at *p*H 10.2 along the different sites of sewage effluent (Fig. 1). The reciprocal relationship between the survival period of fish and the ratio of ammonium:ammonium hydroxide (Fig. 8) suggests that high concentration of ammonium hydroxide generated at high *p*H was the main stressor of the fish mortality. In general, fish are known to survive within the *p*H range of 6.0 to 9.0, but their quality of life is best suited between a *p*H range of 7.0 and 8.0[17].

Inhibition of stress marker enzymes could be considered as important markers to indicate the state of fish health and their physiological conditions. In the present study, *p*H induced increase in both the SDH and alkaline phosphatase activities in gills and liver of the test fish tended to reduce with decline in *p*H of sewage water and corresponding increase in their survival hour (Figs 2-5). An upsurge of these enzymes prior to death against different *p*H conditions (Figs 6-7) clearly indicates the severe stressed condition of the fish causing their instant death. This shows that fish were unable to bear excessive *p*H stress and their physiology failed to acclimatize with the new condition in a very short period. This finally resulted in the offshoot of these stress marker enzymes. This eventually resulted in the offshoot of these stress marker enzyme in the tissues of fish prior to death. In contrast, the percent inhibition of SDH activity of fish over the control increased with rise in acclimatization period of fish (Fig. 9). Ramkritinan *et al.*[18], reported significant decrease in the activity on succinate dehydrogenase activity in muscle, liver and brain tissue of fish *Cyprinus carpio* exposed to different sublethal concentrations of distillery effluent and concluded that the respiratory process in *Cyprinus carpio* shifted under distillery effluent stress resulted in a shift towards anaerobiosis at organ level during sub lethal intoxication.

The non-specific alkaline phosphatase in different tissues of fish recorded prior to death was found to be the direct function of the concentration of phosphate of water (Fig. 10). Though the phosphatase enzyme in

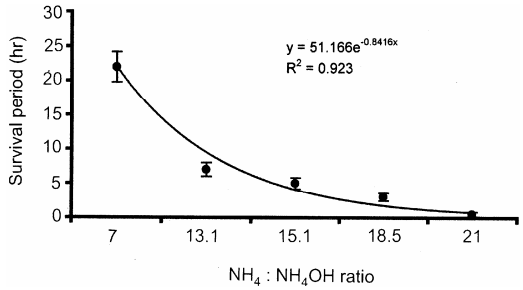

Fig. 8 — Stress induced increase in SDH activity in gills (A) and liver (B) as direct function of acclimatization period of fish exposed to *p*H stress. Percent of increase in SDH activity under stress condition (Experiment-1) was calculated over the data obtained under improved conditions (Experiment-2). Bar indicates the mean (± SE) of SDH activity. Exponential equation was significant at $P < 0.05$.

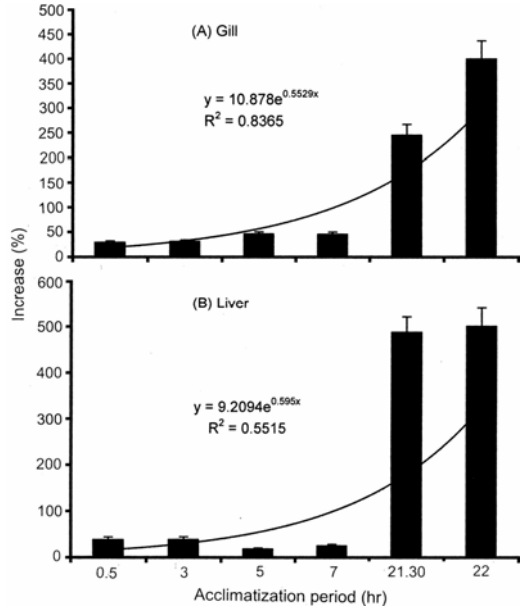

Fig. 9 — Stress induced increase in SDH activity in gills (A) and liver (B) as direct function of acclimatization period of fish exposed to *p*H stress. Percent of increase in SDH activity under stress condition (Experiment-1) was calculated over the data obtained under improved conditions (Experiment-2). Bar indicates the mean (± SE) of SDH activity. Exponential equation was significant at $P < 0.05$.

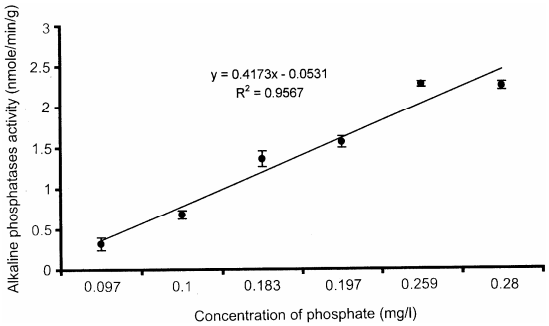

Fig. 10 — Alkaline phosphatases activity as a direct function of ambient phosphate concentrations in different sites of facultative pond. Exponential equation was significant at $P < 0.05$.

primary producers was, in general, used to indicate the P-stress in the ambient environment[3], the direct relationship between the non-specific alkaline phosphatase in the test fish and phosphate concentration of water was mainly due to enrichment of phosphate in the waste water system rather than P-deficiency.

From the results it may be concluded that the analysis of SDH activity of fishes living in the wastewater fed system can effectively be used as an indicator of fish health. Similar analysis of non-specific alkaline phosphatases activity can be used to decide the phosphate status of the ambient environment.

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